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Quantification of Protein Signatures in Archived Human Prostate Tissues Using Shotgun Proteomic Methods

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Biomarkers that robustly predict the metastatic potential of localized CaP are sorely needed to effectively treat localized CaP patients that pose the greatest risk of developing significant CaP. Biomarkers specific to significant CaP are also necessary if more effective drugs are going to be developed that can target and cure patients afflicted by this deadly disease. Proteins represent some of the most powerful molecular biomarkers to human disease such as cancer. Therefore this proposal will implement state-of-the-art methods in biological mass spectrometry to identify protein biomarkers specific to non-significant and significant CaP. These new protein biomarkers may spur the development of molecular tests that robustly predict the metastatic potential of non-significant CaP. These tests would reduce the physical and mental burdens associated with the overtreatment of patients afflicted by localized CaP. Also, protein biomarkers specific to significant CaP may represent new and effective drug targets to cure patients already afflicted by this deadly disease. We anticipate this proposal will identify the critical molecular targets with the greatest potential to improve the treatment and potentially cure CaP in men.

15. SUBJECT TERMS
No subject terms provided.

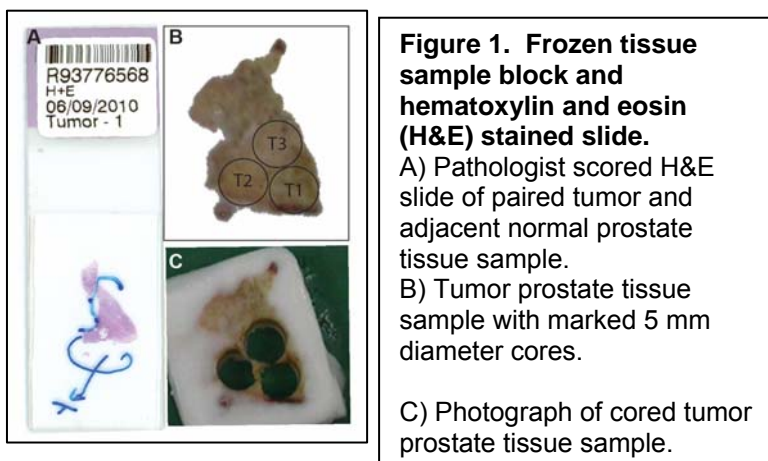| 16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
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INTRODUCTION
Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

My laboratory is currently constructing protein expression libraries of matched normal and cancerous human prostate tissues using quantitative mass spectrometry. Our research progress over the past 12 months has entailed the processing of archived frozen tissues of normal, T2, and T3 staged prostate tissues for proteomic analyses using label-free, quantitative mass spectrometry. We have acquired tissue blocks of whole prostate radial prostatectomies and have focused on optimizing protocols to extract and profile proteins in matched normal and diseased tissue samples using directed mass spectrometry methods. The ultimate outcome of these efforts will be the identification of pathologically-staged protein biomarkers to organ-confined and metastatic human prostate cancers.

BODY
This section of the report shall describe the research accomplishments associated with each task outlined in the approved Statement of Work. Data presentation shall be comprehensive in providing a complete record of the research findings for the period of the report. Provide data explaining the relationship of the most recent findings with that of previously reported findings. Appended publications and/or presentations may be substituted for detailed descriptions of methodology but must be referenced in the body of the report. If applicable, for each task outlined in the Statement of Work, reference appended publications and/or presentations for details of result findings and tables and/or figures. The report shall include negative as well as positive findings. Include problems in accomplishing any of the tasks. Statistical tests of significance shall be applied to all data whenever possible. Figures and graphs referenced in the text may be embedded in the text or appended. Figures and graphs can also be referenced in the text and appended to a publication. Recommended changes or future work to better address the research topic may also be included, although changes to the original Statement of Work must be approved by the Army Contracting Officer Representative. This approval must be obtained prior to initiating any change to the original Statement of Work.

Task: *Specific aim1: Construction of mass spectrometry-based protein expression libraries of normal prostate tissue, non-significant CaP tissue, and significant CaP tissue.*

Figure 1. Frozen tissue sample block and hematoxylin and eosin (H&E) stained slide.
A) Pathologist scored H&E slide of paired tumor and adjacent normal prostate tissue sample.
B) Tumor prostate tissue sample with marked 5 mm diameter cores.

C) Photograph of cored tumor prostate tissue sample.

Over the past year my laboratory has acquired 5 frozen radical prostatectomy (RP) samples containing T2 stage prostate cancer and normal adjacent tissue to build protein expression signatures of non-significant (organ-confined) and significant (metastatic) human prostate cancer using label-free, quantitative mass spectrometry. Last year we were able to extract up to 100 micrograms of total protein using whole-mount FFPE RP tissue block samples. This

was a significant improvement in the amount of extractable protein that was isolated in needle-biopsied tissue cores in the first year of this research application (*e.g.* 1-5 ug of total protein). We have been able to increase the amount of extracted protein up to 1 mg of N-linked glycoproteins using frozen whole-mount tissue blocks (**Fig. 1**). This has greatly enhanced our ability to interrogate the protein expression patterns in no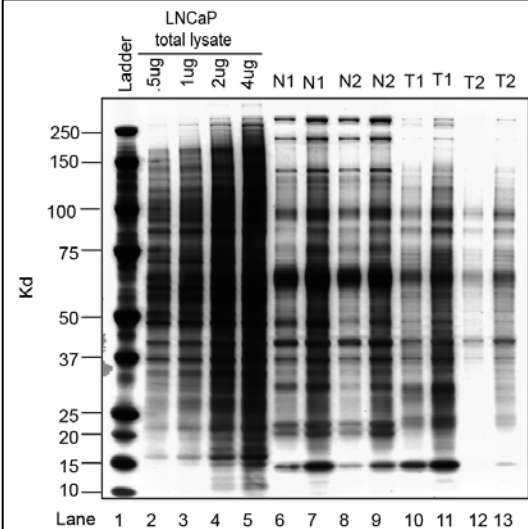n-significant and significant human prostate cancers by nanospray liquid-chromatography tandem mass spectrometry (nano-LC-MS/MS). The frozen tissue blocks provide up to 500X more material to identify human prostate cancer protein biomarkers.

Figure 2. Silver-stained SDS-PAGE gel of total protein extracted from sample R90197381. Detergent solubilized proteins were run into lanes 6-13. A protein standard composed of LNCaP whole cell lysates at .5ug, 1ug, 2ug, and 4ug was loaded into lanes 2-5. Protein isolated from normal adjacent tissues, samples 1 and 2, were loaded into lanes 6-9 (sample 1, lanes 6-7; sample 2, lanes 8-9). Protein isolated from tumor tissues, samples 1 and 2, were loaded into lanes 10-13 (sample 1, lanes 10-11; sample 2, lanes 12-13). 1 (lanes 6, 8, 10, and 12) and 2 (lanes 7, 9, 11, and 13) microliters of sample were subjected to SDS-PAGE.

My laboratory has also optimized a new protein-extraction protocol to interrogate glycoproteins in frozen tissue samples using lectin-affinity chromatography methods. This glycoprotein enrichment protocol facilitates the extraction of N-linked glycoproteins in tissue and facilitates the proteomic interrogation of low-abundance biomarkers in tissue samples.
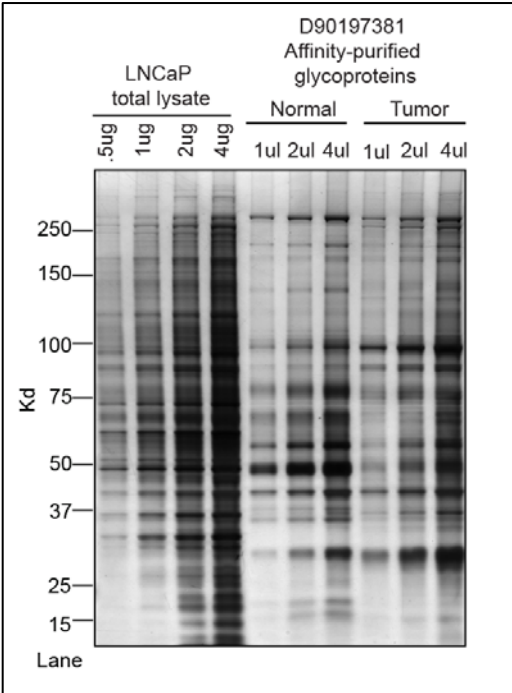
Briefly, tissue samples were extracted with detergent and processed by SDS-PAGE to determine the quantity and quality of tissue extracted proteins (**Fig. 2**). The detergent-solubilized proteins were subsequently incubated with wheat-germ agglutinin (WGA) and concanavalin-A (ConA) beads, washed, and eluted with soluble n-acetyl glucosamine. The eluted glycoproteins were dialyzed to remove residual detergent, and both the integrity and quantity of glycosylated protein samples was determined by SDS-PAGE and silver-stain analysis (**Fig. 3**). Equal amounts of tumor and normal adjacent sample were digested with trypsin and subjected to strong cation exchange chromatography (**Fig. 4**). The individual peptide fractions were collected, desalted, and subjected to nano-LC-MS/MS. This protocol facilitates the mass spectrometry analysis of glycoproteins in cancerous lesions of the prostate and adjacent normal tissue. We have preliminary MS data showing the differentially expression of glycoproteins such as prostate-specific antigen (PSA) and prostatic specific phosphatase (PSAP), which are well-known androgen-regulated glycoproteins and biomarkers used in the detection of localized prostate cancers (*1*).

Figure 3. Silver stain of affinity-purified glycoproteins from tumor and normal adjacent prostate tissue (sample D90197381).

To expedite the discovery of pathologically-staged protein biomarkers to non-significant and significant human prostate cancer in archived tissue samples, last year my laboratory implemented a new mass spectrometry workflow called "Directed Proteomics"(*2*). In contrast to shotgun proteomic methods, which attempts to sequence all ionized tryptic peptides in a complex samples using data-dependent (DD) acquisition methods, we have instead opted for a mass spectrometry profiling strategy to detect and sequence peptide ion differences across multiple tissue samples. This directed proteomic approach have been superior to our past shotgun proteomic experiments. This reflects our ability to sequence lower intensity peptide ions in the complex tissue sample relatively to shotgun proteomic protocols that routinely selects high-abundant peptide ions for MS/MS. We are in the process of submitting a manuscript detailing the strengths of this new data-acquisition scheme to identify and quantify protein biomarkers in archived tissue samples shortly.

Last year, my laboratory established a new collaboration with Dr. Michael B. Cohen, the departmental head of Pathology at the UI Carver College School of Medicine. We have continued our collaboration with Dr. Cohen over the past year, as his laboratory has provided pathological expertise in the annotation of archived frozen radical prostatectomy tissue blocks. Dr. Cohen's has volunteered his time to help my laboratory carefully annotate the Gleason score of non-significant and significant human prostate cancer samples.

We will complete the processing of cancerous and normal tissue samples over the next year, as these samples will allow us to build comprehensive protein expression libraries of normal prostate tissue, non-significant prostate cancer tissue, and significant prostate cancer tissue.
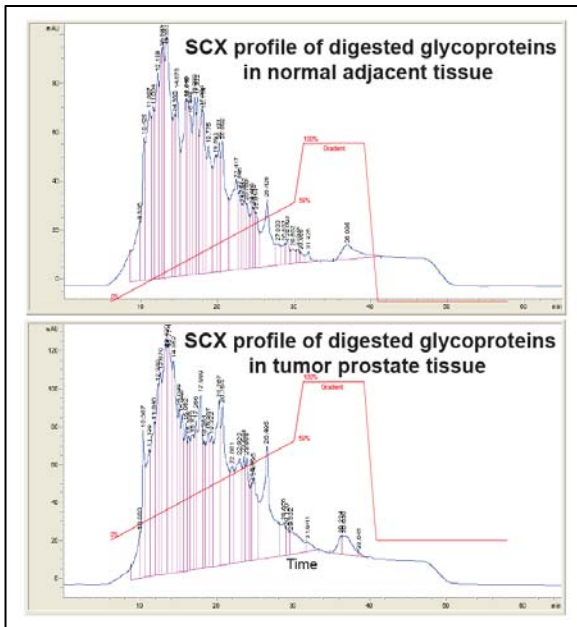

Figure 4: U.V. trace chromatogram of strong cation exchanged (SCX) fractionated peptides of affinity-purified glycoprotein tissue samples of paired normal and tumor tissue samples. Each sample was subjected to a 65 minute gradient of 0-59% buffer B (300 mM KCl) and peptides were collected every 1 min for 40 min for a total of 40 SCX fractions.

Task: *Specific aim 2: Identification of protein biomarkers in non-significant and significant CaP tissues using novel statistical methods.*

We are in the process of using pathway and network analysis tools (*e.g.* GeneGo) to identify differentially expressed protein biomarkers in NAT and CAN tissue samples. As shown in **Fig. 5**, *Genego* pathway analysis of sample R93776568 showed the overexpression of glycoproteins involved in ER-Golgi trafficking and protein glycosylation in CAN relative to NAT. These preliminary findings would suggest the upregulation of biosynthetic pathways in the CAN tissue as have been noted previously (4). We have continued to work with LabKey to store and analyze our MS data in Computational Portal Analysis System (CPAS) database to identify statistically significant protein biomarkers of non-significant and significant human prostate cancer samples.

KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research.

• My laboratory is generating glycoprotein expression profiles of normal adjacent prostate tissue, non-significant prostate cancer tissue, and significant prostate cancer tissue using directed proteomic methods.
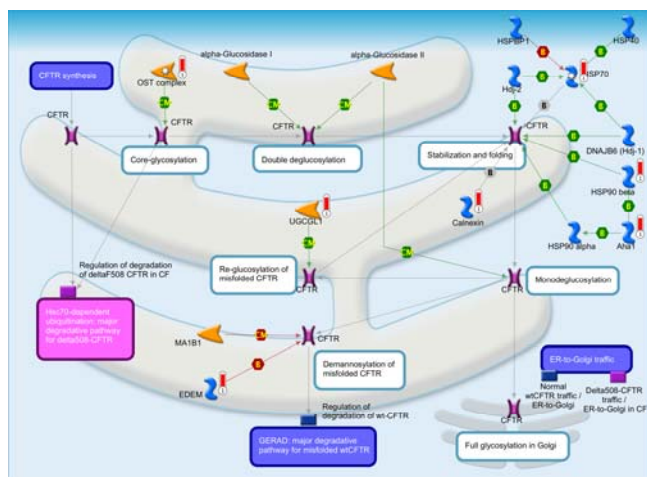

Figure 5. Network pathway overexpressed in CAN in sample R93776568. Genego pathway analysis identified proteins involved in ER-Golgi trafficking and CFTR synthesis significantly (*i.e.* p-value < 7.0 x 10^{-7}) upregulated in CAN versus NAT.

• We have developed a glycoprotein tissue-extraction protocol has been developed for the proteomic analysis of archived frozen tissue samples.

• We have successfully implemented a directed proteomic approach that has significantly improved our ability to identify and quantify protein expression changes across normal prostate tissue, non-significant prostate cancer, and significant human prostate cancer.

Addendum: Previous report was unacceptable because it did not have reportable outcomes in context to the Statement of Work.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

Statement of Work

Specific aim 1: Construction of mass spectrometry-based protein expression libraries of normal prostate tissue, non-significant CaP tissue, and significant CaP tissue.

This task involves constructing protein expression libraries of normal prostate tissue, non-significant CaP tissue, and significant CaP tissue using the DTP method. More specifically, 15 independent radical prostatectomized, needle-biopsied formalin-fixed paraffin-embedded tissue samples (52 μm x 2 mm sections) representing either normal prostate tissue, non-significant (T2) CaP, or significant (T3/T4) CaP tissues will undergo shotgun proteomic analysis to quantify protein expression in each sample. The estimated timeline for the analyses is shown below:

Expected outcomes: We anticipate each protein library will contain the relative expression of ~800-1,000 proteins.

Reportable outcomes for 2011-2012: *Over the past year my laboratory has processed **4 out of a total of 15 radical prostatectomized tissue samples** for proteomic analyses using directed MS. We*
have completed a preliminary expression analysis of lectin-affinity purified glycoproteins between cancerous and normal adjacent tissue samples. As shown in Table I, we were able to quantitatively profile glycoprotein expression in normal adjacent tissue (NAT) and cancerous (CAN) tissue samples. Our initial analyses have quantified expression differences in N-linked and O-linked cell surface receptor

Table I. Quantification of Glycoproteins in NAT and Cancerous Tissue Samples.

Sample	NAT	CANCER	Overlap	NAT Unique	CANCER Unique
R03188233	1,034	1,572	677	357	895
R93776568	942	1,180	615	227	565
R90197381	1,583	1,857	926	657	931
R42521246	1,263	356	275	988	81

Total non-redundant protein identifications (IDs) quantified using directed MS at a ≤ 1% FDR.

glycoproteins and intracellular O-linked (e.g. N-acetyl-D-glucosamine, GlcNAc) glycoproteins. We have not yet completed the proteomic analysis of NAT and CAN with the complete set of 15 tissue samples, but we anticipate the complete proteomic analysis of by the end of the next reporting period.

Modification of protein extraction protocol:

We have slightly modified the protein extraction protocols to proteomically process isolated glycoproteins from frozen radical prostatectomy tissue samples. Our original method of processing formalin-fixed paraffin-embedded tissue samples using shotgun proteomics was less efficient method for quantifying differentially expressed proteins between samples. As shown in Table I, the new proteomic approach has increased the proteomic coverage of our analyses relative to shotgun proteomic analysis of formalin-fixed tissue samples (unpublished observations). Therefore we will processing the15 frozen radical prostatectomy samples of NAT and CAN to develop mass spectrometry-based protein expression libraries of normal, non-significant, and significant CaP tissue.

Specific aim 2: Identification of protein biomarkers in non-significant and significant CaP tissues using novel statistical methods.

This task will involve implementation of statistical tests to identify protein biomarkers unique to non-significant (T2) and significant (T3/T4) CaP tissue. The proposed analyses are expected to occur over all three years with the bulk of the statistical analyses to occur during years 2 and 3.

Expected Outcomes: *We anticipate the identification of specific protein biomarkers to non-significant and significant CaP tissues.*

Reportable outcomes for 2011-2012: *We are actively uploading, storing, and annotating differentially expressed between NAT and non-significant (T2) and significant (T3/T4) CaP tissues using the Computational Portal Analysis System (CPAS). We have yet to carry out a detailed statistical analysis of differentially expressed proteins between samples because samples have yet to be completely processed by MS analyses. We anticipate by the end of the next reporting period that our analyses will have identified a subpopulation of specific protein biomarkers to non-significant and*

significant CaP tissues. We have initiated preliminary bioinformatic analyses to identify differentially expressed proteins between NAT, non-significant, and significant tissue samples (Fig. 5).

REFERENCES: List all references pertinent to the report using a standard journal format (i.e. format used in *Science, Military Medicine,* etc.).

1. J. Tosoian, S. Loeb, PSA and beyond: the past, present, and future of investigative biomarkers for prostate cancer. *ScientificWorldJournal* **10**, 1919 (2010).
2. A. Schmidt, M. Claassen, R. Aebersold, Directed mass spectrometry: towards hypothesis-driven proteomics. *Curr Opin Chem Biol* **13**, 510 (Dec, 2009).

APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

None reported to date.

SUPPORTING DATA: All figures and/or tables shall include legends and be clearly marked with figure/table numbers.

Data analyses are not complete. Completed datasets will be provided over the final reporting period.